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14. ABSTRACT:
The goals are 1) to define vulnerability of the embryonic heart to oxygen (O2) deprivation 2) to determine if placental insufficiency induced by knock-out of HIF1a in the mother (MATcKO) increases this vulnerability and how. We used the ODDLuc hypoxia reporter as a sensitive read-out of tissue oxygenation. We have found that even moderate acute reductions in maternal inspired O2 (to 12%, room air is 21%) induce a hypoxic response in the embryonic heart at mid-gestation. During the remainder of the funding period we will be testing milder hypoxic conditions (to 18%) at different stages of development. For the 2nd aim we have found that MATcKO HIF1a causes a number of defects in the developing placenta, including reduced invasion by uterine natural killer cells (uNK) and conceptus-derived trophoblast cells. The placental defects do not alter basal O2 delivery to the embryo but make it more vulnerable to O2 deprivation. Some embryos are non-viable at E15 with abnormal hearts but do not model human CHD. Further experiments will examine stage–dependent effects of O2 deprivation in this model.
The developing fetal heart is vulnerable to even mild O2 deprivation at mid-gestation, and this may be compounded by placental defects.

15. SUBJECT TERMS: Hypoxia, Hypoxia Inducible Factor, Placenta, Congenital Heart Defects, Spiral arteries, Trophoblast, Fetal-placental unit, Decidua

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1. INTRODUCTION

The number of women of child bearing age in active duty in the armed services is increasing. Approximately 10% of these women will have unplanned pregnancies and thus may not recognize that they are pregnant until the second or third month. These women in active duty are sent to environments that they would not experience at home such as high altitude (HA). It is well established that the chronic reductions in oxygen (hypoxia) at high altitude adversely affects the pregnancy and developing fetus, manifest as pre-eclampsia and intra-uterine growth restriction (IUGR), respectively. The idea for this proposal is that acute HA hypoxic exposure early in pregnancy during a critical period of organogenesis may critically reduce O2 transport to the fetus thereby causing Congenital Heart Defects in at-risk pregnancies.

This project uses novel mouse models to test this idea. The first objective is to use a novel reporter of O2 concentrations, the ODDLuc mouse, to determine the dose-response relationship between HA hypoxia and reduced O2 delivery to the fetus as a function of developmental stage. These mice contain a novel reporter of [O2], ODD-Luciferase, in which the Oxygen Degradation Domain of *Hif-1α* (residues 530-652, spanning the Proline564 hydroxylated by prolyl hydroxylase (PHD) was fused upstream of the firefly Luciferase (Luc) coding sequence and incorporated into the *Rosa26* locus. Timed pregnant ODDLuc mice are acutely exposed to O2 concentrations ranging from 8% (equivalent to 25000 ft elevation) to 18% (equivalent to 4000 ft elevation) at early (E9-11), mid (E13-15) or late (E18) stages of their pregnancies and luciferase activity measured in fetal heart and other tissues. This will determine at what altitude and developmental stage O2 delivery to the developing heart is compromised and by how much.

The second objective is to develop a novel mouse model of placental insufficiency to examine how maternal-fetal and gene-environment interactions may influence the effect of HA hypoxia on the developing heart. To accomplish this hypoxia-Inducible transcription factor HIF-1a is conditionally inactivated in the maternal decidual contribution to the placenta using a Tamoxifen-inducible Cre-lox strategy. This is predicted to suppress hypoxia-dependent remodeling of the maternal decidua and reduce the mother's ability to supply O2 to the developing fetus. ODDluc in these mice is used to report on the effect on O2 delivery to the fetal heart in this model of placental insufficiency and its exacerbation by high altitude hypoxia.

We expect to show that the developing heart is particularly sensitive to reductions in O2 supply early in development during the critical stage of organogenesis. We expect that placental insufficiency and reductions in O2 during this stage will cause common CHDs such as Ventricular Septal Defect and conotruncal heart defects, for example Tetralogy of Fallot . If correct this would provide substantial information as to how to advise and restrict military service at high altitude in those women who are or may become pregnant.

2. KEYWORDS

Hypoxia, Hypoxia Inducible Factor, Placenta, Congenital Heart Defects, Spiral arteries, Trophoblast, Fetal-placental unit, Decidua, cell proliferation, Bioluminescence imaging, Luciferase.

3. ACCOMPLISHMENTS

➢ What were the major goals of the project?

Goals were classified into 2 specific aims and sub tasks. The percent work completed are described below in Table 1 and Table 2:

Specific Aim 1: ODDLuc as a novel indicator of the effect of high altitude hypoxia on placental O_2 transport and fetal O_2 metabolism

Table 1. Measurement of ODD Luciferase activity in fetal tissues	Time line (Months)	% completed
Subtask 1 Expand colony of ODDLuc mice	1-3	100
Subtask 2 Measurement of ODD Luciferase activity in tissue homogenates form timed pregnant mice after hypoxic exposures 5 pregnant ODDLuc mice per group x 35 groups (5 developmental stages x 7 O2 concentrations) = 175 mice	3-6	45
Subtask 3 Bioluminescent imaging of ODD Luciferase activity in intact embryos and sections after hypoxic exposures	5-6	0
Milestone(s) Achieved: Dose-response of maternal hypoxic exposure on oxygen delivery to fetal heart + other tissues as a function of developmental stage	8	45
Local IACUC Approval	1-2	100
Milestone Achieved: HRPO/ACURO Approval	1-2	100

Specific Aim 2: Conditional KO of HIF-1a in maternal decidua as a novel model of placental insufficiency and O_2 deficit in Congenital Heart Defects:

Table 2. Major Task 2 Novel mouse model of placental insufficiency + hypoxic exposure- effect on O2 metabolism and Congenital Heart Defects	Time line (Months)	% completed
Subtask 1 Expand colony of Cre+//f/f mice needed for model of maternal cKO of HIF-1α BACTCreTm+//f/f females mated with f/f males	8-12	100
Subtask 2 Treat timed pregnant mice with Tamoxifen to inactivate HIF-1a and expose mice to hypoxia BACTCreTm+//f/f timed pregnant females are mated with ODDLuc males + treated with Tamoxifen (3 mg/40g) at E8.5 (see Subtask #3 for # mice)	12-18	60
Subtask 3 Timed pregnant mice from Subtask 2 are exposed to hypoxia at E11 or E15 5 pregnant mice per group x 8 groups (2 developmental stages x 4 O2 concentrations) = 40 pregnant mice	12-18	50
Subtask 4 Morphologic and histologic analysis of fetal hearts and placentas	14-18	100
Subtask 5 Measurement of ODD Luciferase activity in and bioluminescent imaging of fetal tissues	14-18	50
Milestone(s) Achieved: Determination of effect of placental insufficiency coupled with oxygen deprivation on fetal heart and placental oxygen metabolism and structural heart defects. *Presentation at 2 national meetings and publication of 1-2 papers*	18	70

➢ What was accomplished under these goals?

SPECIFIC AIM 1: ODDLuc as a novel indicator of the effect of high altitude hypoxia on placental O_2 transport and fetal O_2 metabolism

Subtask 1 Expand colony of ODDLuc mice:

ODDLuc males and females were purchased from Jackson laboratory (stock # 006206). This mouse contains the Oxygen Degradation Domain (ODD) of *Hif-1α* fused to N-terminus of the Luciferase (Luc) coding sequence expressed from the *Rosa26* locus in which the fusion protein 'ODD-Luc' expressed constitutively under O2 deprivation. Homozygous ODD-Luc females crossed with ODDLuc males to expand the colony. All the offspring obtained from this cross were homozygous for ODDLuc.

We requested for a total of 175 ODD-Luc females for O2 dose response experiments proposed in specific aim 1 (Table 3). Currently, we have utilized 60 mice to expose to 8 and 12% O2, and 15 mice for ongoing experiments in which mice were exposed to 16% O2 (highlighted in table 3). We are maintaining 4 OD-Luc breeding pairs, which will generate approximately 100 mice to be used in experimentation over the next 5 months. These mice are sufficient to complete the proposed experiments in specific aim 1.

Table 3. Experimental groups proposed to Specific aim 1

Gestation day	**O_2 Concentrations (%)**						
	Control (Room air-21%)	**8**	**10**	**12**	**14**	**16**	**18**
E9.5	5	5	5	5	5	5	5
E11.5	5	5	5	5	5	5	5
E13.5	5	5	5	5	5	5	5
E15.5	5	5	5	5	5	5	5
E18.5	5	5	5	5	5	5	5
TOTAL # mice	**175**						

Subtask 2

Measurement of ODD Luciferase activity in tissue homogenates from timed pregnant mice after hypoxic exposures:

Specific Objectives: To measure the effect of O2 deprivation (Hypoxic stress) and threshold at which oxygen delivery is compromised to the developing embryo and fetus at specific stages of

development. We used ODDLuc mice as quantitative indicator of O2 deprivation in the embryonic/fetal tissues and placenta.

Major activities: Adult ODDLuc females were crossed with ODDLuc males, the day vaginal plug found was considered as embryonic day (E) 0.5. Timed pregnant females of gestational age E9.5, 10.5, 11.5, 13.5, 15.5, 17.5, were placed in the hypoxia chamber, exposed to reduced O2 concentration of 8% for 4hrs, or 12 % x 4-8 hrs, or 16% x 8 hrs (Table 3). For control, pregnant dams were placed in the similar chamber with 21% O_2 (Room air). Luciferase activity was measured in placental and fetal tissue lysates according to manufacturer's instructions (Promega). Activity was normalized to total protein and expressed as fmol luciferase/mg protein or as fold-change vs developmental age-matched control.

Significant results: Reduction of the mother's inspired O2 to 8% or 12% both significantly increased ODDluc activity in the embryonic heart (Fig. 1, p.16; Fig. 2 p.17). This was true at E11.5, prior to maturation of the placenta for O2 exchange, and after, at E15.5. The induction by 8% O2 was significantly greater than with 12% (compare Figs. 1+2), indicating a dose-response. The relative induction of ODDluc was similar at E11.5 and 15.5. However because the basal ODDLuc activity was greater at E11.5, the absolute induction of ODDLuc was greater at the earlier time point. This suggests that the O2 reserve, and thus vulnerability to O2 deprivation, is greater earlier in development, before the placenta is functionally mature. The other fetal organs showed responses that were similar to that of the heart (Figs1,2).

We have exposed pregnant dams to more mild O2 deprivation (16%) and are in the process of measuring ODDluc activity in the fetal tissues. We also are planning to complete O2 dose-response against developmental time points as indicated in Table 3.

Other achievements: None

Goals not met: In Table 3, the experiments completed or completed but waiting analysis are highlighted in yellow. We have not completed the full range of O2 dose response experiments. We have ongoing breedings to generate required number of ODD-Luc females (100) to complete full range of O2 dosing experiments against gestational stage during the last six months of funding. We do not anticipate any problems to complete experiments proposed in specific aim 1.

SPECIFIC AIM 2: Conditional KO of HIF-1a in maternal decidua as a novel model of placental insufficiency and O_2 deficit in Congenital Heart Defects

Subtask 1+2 Expand colony of Cre+//f/f mice needed for model of maternal cKO of HIF-1α BACTCreTm+//f/f females mated with f/f males

Hif-1α$^{f/f}$ mice (stock # 007561) and Tamoxifen (TM) inducible β-actinCre^{+} (CAGGCre-ER^{TM}; stock # 004682) were procured from Jackson laboratory. β-actinCre^{+} mice were crossed with Hif-1α$^{f/f}$ mice to obtain Hif-1α$^{f/f}$,β-actinCre^{+} females.

	Table 4. Experimental groups for Specific Aim 2, sub task 2+3			
Groups/Genotype	# of mice E11.5	E13.5	E15.5	O_2 Concentration (%)
HIF-1α +/+, Cre+	5	5	5	**21 (room air)**
HIF-1α f/f, Cre+	5	5	5	**8**
	5	5	5	**12**
	5	5	5	**16**
TOTAL # mice		**60**		

Table 5. Number of mice required for sub task 4	
Groups/Genotype	Number of mice
HIF-1α +/+, Cre+	3
HIF-1α f/f, Cre+	3
TOTAL # mice	**6**

Subtask 2 Treat timed pregnant mice with Tamoxifen to inactivate HIF-1a and expose mice to hypoxia. BACTCreTm+//f/f timed pregnant females are mated with ODDLuc males + treated with Tamoxifen (3 mg/40g) at E8.5

Subtask 3 Timed pregnant mice from Subtask 2 are exposed to hypoxia at E11 or E15

5 pregnant mice per group x 8 groups (2 developmental stages x 4 O2 concentrations) = 40 pregnant mice

Specific Objectives: To determine if placental insufficiency increases the vulnerability of the embryo/ fetus to O2 deprivation. To accomplish this we inactivated HIF-1a in the maternal cells and exposed pregnant dams to varying concentrations of O2 (see Table 4).

Major activities: *Hif-1α$^{f/f}$,β-actinCre^{+}* females were mated with ODD-Luc males and treated with TM at E8.5-9.5. Control mice were *Hif-1α$^{+/+}$,β-actinCre^{+}* similarly treated with TM. Pregnant dams were treated with TM (3mg/40g bw; Sigma # T5648) in sunflower oil on E8.5 and 9.5 to induce the activity of Cre recombinase. Mice were genotyped by standard PCR methods using primers as described on the Jackson Laboratory website. PCR of genomic DNA was performed to test Cre efficiency using forward 5' GGATGAAAACATCTGCTTTGG 3' and reverse 5' ACTGCCCCAACACAATACTTTT 3' primers. Recombination of *Hif-1α* was ~90% in the MATcKO placentas of Tamoxifen-treated mice measured at E15.5 vs ≤20% in placentas of untreated (control) mice of the same genotype.

Significant results: Assays for these experiments were sub-tasks 4+ 5; the data are presented in these sections below.

Other achievements: None

Goals not met: In Table 4, the experiments completed are highlighted in yellow. We have not completed the full range of O2 dose response experiments x developmental stage. We have ongoing breedings to generate required number of females to complete full range of O2 dosing experiments against gestational stage during the last six months of funding. We do not anticipate any problems to complete experiments proposed in specific aim 2.

Subtask 4: Morphologic and histologic analysis of fetal hearts and placentas:

Specific Objectives: To determine if MATcKO HIF-1a causes heart and placental defects.

Major activities: *Hif-1α$^{f/f}$,β-actinCre^{+}* females were mated with ODD-Luc males and injected with Tamoxifen (3 mg/40 g bw) on E8.5 and 9.5. Control (CON) pregnant mice were *β-actinCre^{+}* and treated with Tamoxifen in the same manner. Pregnant dams were euthanized by CO_2 inhalation on E15.5, embryos and placentas collected and fixed in 4% paraformaldehyde on E13.5 or E15.5. Paraffin embedded fetal and placental sections were deparaffinized and rehydrated through a series of ethanol and subjected to Hematoxylin and Eosin stain. Placental sections were further analyzed by immunohistochemical and in situ hybridization assays.

Significant results:

Effect of MATcKO on fetal development: In these MATCKO experiments the embryos would be either of 2 genotypes: HIF-1α$^{f/+}$,β-actinCre^{+} embryo are designated E:Cre+ and HIF-1α$^{f/+}$,β-actinCre^{-} embryo as E:Cre-. The former are effectively heterozygous null for HIF-1a, while the latter are effectively wild type. Embryos of each genotype were separately analyzed. Embryos from MATcKO dams were harvested on E15.5 and analyzed in whole mount and section.

MATcKO E:Cre- embryos had normal appearance and viability while MATcKO:E:Cre+ embryos did not. AT E15.5 most of these embryos (96%) were non-viable and slightly smaller than their littermates. In whole mount these embryos showed severe hemorrhage, edema, and reduction in blood-filled vessels on the surface of the embryo (Fig.4, p.21). In H&E stained sections the hearts of these embryos were delayed in their development. The basic structures and anatomic relationships were normally present, such as the 4-chambered heart, the great vessels (aorta, pulmonary arteries) and their anatomic relationships. A notable abnormality in the MATcKO E:Cre^{+} was the marked thickening of the epicardium. There also appeared to be abnormal persistence of mesenchyme in the atrial and ventricular septum. Whether these and other differences represent developmental delay, are secondary to the non-viability that develops between E13.5-15.5, or are specific to the MATcKO synergizing with embryonic effective heterozygosity for Hif-1α, will require further study.

Effect of MATcKO on placental development and morphology: Placentas were examined at E13.5 and E15.5 when placental and fetal organ morphogenesis are nearing completion. At E15.5 MATcKO E:Cre^{+} placentas were visibly smaller and weighed ~15% less than CON while MATcKO E:Cre^{-} placentas were unchanged (CON: 98.1±2.0mg; MATcKO E:Cre^{+}: 84.4±1.5mg, P<0.001; MATcKO E:Cre^{-}: 104.7±2.83mg, p=0.08).

The general appearance of MATcKO placentas was normal while a number of abnormalities were evident in histological assays. Particularly striking in MATcKO placentas were reductions in the numbers of cells migrating into the maternal decidual portion of the placenta.

Uterine Natural Killer (uNK) cells were identified by Perforin (Prf) immunostaining. These maternally derived immune cells are recruited to the maternal decidua and are thought to play a role in the remodeling of the maternal spiral arteries. The number of Prf+ uNK cells was markedly reduced in the MATcKO decidua at E13.5 and E15.5 (Fig.5). At higher magnification, uNK cells could be seen to encircle the vessels within the decidua of control placentas, while in the MATcKO the uNK cells did not appear to localize to any particular anatomic structure.

Trophoblast Cells were identified by a number of markers including *Tpbpa, Plp-F (Prl7a2), Plp-N (Prl7b1), Plf (Prl2c2), Gcm1* and *Syna* mRNAs, by *in situ* hybridization. *Tpbpa* is a marker of spongiotrophoblasts (Sp-T) and glycogen trophoblasts (Gly-T), *Plp-F* marks Sp-T only, and *Plp-N* identifies Gly-T cells in all junctional zone subtypes.

Plf (*Prl2c2*) identifies parietal trophoblast giant cells (p-TGCs) located at the border of the fetal placenta and decidua, and spiral artery trophoblast giant cells (SA-TGCs) associated with spiral arteries (SA) in the maternal decidua, where they participate in arterial remodeling. *Plf* (*Prl2c2*)-positive trophoblast cells were observed in E15.5 CON and MATcKO placentas at the border of the maternal decidua and fetal placenta (Fig. 6). At higher magnification, the association of TGCs with maternal decidua SAs appeared to be reduced in the MATcKO placentas (Fig. 6C, D, arrows). Furthermore, *Plf* (*Prl2c2*) staining appeared to be more frequent in the labyrinth layer of MATcKO placentas (Fig. 6A, B, arrows), suggesting aberrant TGC differentiation and/or migration into the labyrinth zone in the placentas of these mice.

In summary these studies suggest that KO of HIF1-a in the maternal decidua (MATcKO) suppresses the recruitment of uNK and TB cells into the maternal decidua. These two cell populations are thought to be required for the remodeling of the maternal decidual spiral arteries (SA) at mid-gestation. This remodeling is required to convert the SAs into flaccid conduits to optimize placental O2 transport to the fetus. Thus it is reasonable to hypothesize that the placental defects induced by MATcKO HIF-1a render the fetus more vulnerable to O2 deprivation, as tested in the next sub-task,.

Proliferation and Apoptosis in the Placenta

Placentas were stained for Ki67 and TUNEL to determine if MATcKO HIF-1a may have secondary effects on the health of the placenta. Ki67 staining, an indicator of cell proliferation, was significantly reduced in the labyrinth of all E15.5 MATcKO placenta (Fig.7). TUNEL positive cells, indicating apoptosis, were increased in the labyrinth of E15.5 MATcKO E:Cre^{+} placentas (Fig. 8). Together this suggests decreased proliferation and increased apoptosis specifically in the labyrinth layer of E15.5 MATcKO placentas as secondary effects causing atrophy of the placenta in the MATcKO HIF-1a model.

Other achievements: None

Goals not met: None

Sub-task 5 Measurement of ODD Luciferase activity in and bioluminescent imaging of fetal tissues

Specific Objectives: To measure the effect of O2 deprivation (Hypoxic stress) and threshold at which oxygen delivery is compromised to the developing embryo and fetus at specific stages of development in MATcKO mice.

Major activities: MATcKO HIF1-a during pregnancy was achieved as described above. Pregnant mice were then exposed to reductions in inspired O2 at E11.5, 13.5 or 15.5 as described in Aim 1. Fetal tissues were harvested and ODDluciferase activity measured as described in Aim 1 and reported as fold-change vs control (control mice breathing room air)

Significant results: MATcKO had no effect on basal ODD-Luc activity in the fetal organs at any of the gestational ages examined (Fig. 3,P.19). MATcKO also did not affect the magnitude of hypoxic induction of ODD-Luc activity in E11.5 heart, liver and brain. In contrast, MATcKO rendered the fetuses more vulnerable to O_2 deprivation at E13.5 and 15.5. At these gestational ages MATcKO synergized with reductions in O_2 concentrations (8% O_2 for 4 hours) to increase ODD-Luc activity in the fetal tissues. The induction of ODD-Luc activity caused by MATcKO and O_2 deprivation at later gestational ages is similar to that caused by O_2 deprivation alone at the earlier stage (E11.5) prior to the maturation and function of the feto-placental unit for O2 transport.

In summary the placental defects induced by MATcKO HIF1a increased the vulnerability of the embryonic heart to O2 deprivation at E13. This is when the feto-placental unit is becoming functional for blood-borne transport of 02 to meet the increasing demand of the developing fetus. This is occurring at a critical stage of heart development when the heart is completing septation between the chambers and the great arteries are establishing their connections with the ventricles (aorta to the left ventricle and pulmonary artery to the right ventricle). These are the most common sites of human CHD and suggest that placental defects and vulnerability to O2 deprivation could play a role in the development of CHD in pregnant women subject to O2 deprivation at critical stages of pregnancy.

Other achievements None.

Goals not met: We have not completed the full range of O2 dose response experiments against different gestational ages as shown in the Table. This will be completed in the remaining time on the grant. We do not anticipate any problems to complete experiments proposed in specific aim 2.

- **What opportunities for training and professional development has the project provided?**

  'Nothing to report'

- **How were the results disseminated to communities of interest?**

  'Nothing to report'

- **What do you plan to do during the next reporting period to accomplish the goals?**

Specific aim 1:

In specific aim 1, sub task 1, we have proposed to measure ODD-Luc activity in pregnant dams of gestational ages E9.5 - E18.5 exposed to O2 concentrations ranging from 8 -18% (see table 3 below). We have completed measurement of ODD-Luc activity in E9.5-E18.5 and fetal tissues of pregnant dams exposed to 8% and 12% O2 for 4hrs. To complete the proposed experiments in specific aim 1, we will expose timed pregnant ODD-Luc mice of gestational ages E9.5 – E18.5 to O2 concentrations ranging 10, 14, 16 and 18% for 4- 8hrs. Fetal and placental tissues will be collected following exposure, ODD-Luc activity measured in tissue lysates as explained above in accomplishments. We have established homozygous ODD-Luc colony and we have ongoing breedings which will generate sufficient number of females and males required for these experiments. At this point we do not anticipate any problems to complete the objectives proposed in sub task 1.

In specific aim 1 sub task 3, we proposed bioluminescent imaging of ODD Luciferase activity in intact embryos and sections after hypoxic exposures. Placentas and embryos (n=3-4) from pregnant dams exposed hypoxia (sub task 1) will be used for bioluminescent imaging in intact embryos and in cryosections.

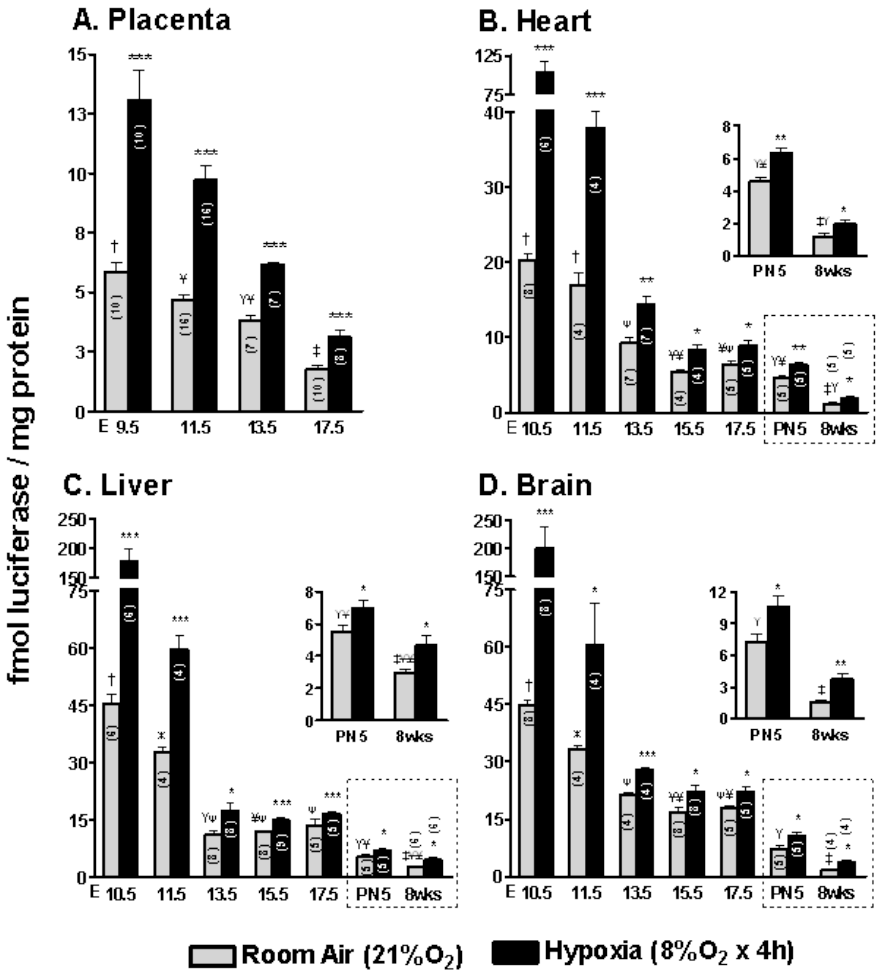
Specific aim 2:

In specific aim 2, we have completed sub task 1, 2 and 4 (see table 2 and Accomplishments). We proposed to measure ODD-Luc activity in MATcKO pregnant dams of gestational ages E11.5 and E15.5 subjected to O2 deprivation (8, 12 and 16%) for 4 hrs. Currently we have tested the effect of 8% O2 on E11.5, E13.5 and E15.5 MATcKO placental and fetal tissues. To complete experiments proposed in specific aim 2, we will expose MATcKO pregnant dams to 16 and 12% O2 for 4 hrs on E11.5, 13.5 or E15.5. Fetal and placental tissues will be harvested to measure luciferase activity. 3-4 placentas from this

experiment will be used for bioluminescent imaging. We have established HIF-1a fl/fl,Cre+ and ODD-Luc colony and we have ongoing breedings which will generate sufficient number of females and males required for these experiments. At this point we do not anticipate any problems to complete the objectives proposed in specific aim 2.

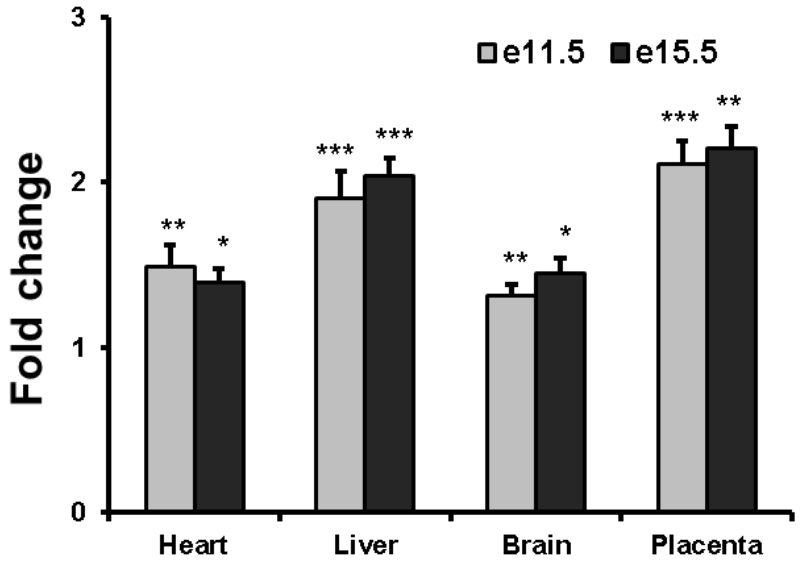
Figures

Figure 1. ODD-Luc activity 1) declines during normal mouse development 2) is induced in fetal organs by reduction in dam's inspired O2 to 8% in proportion to basal activity



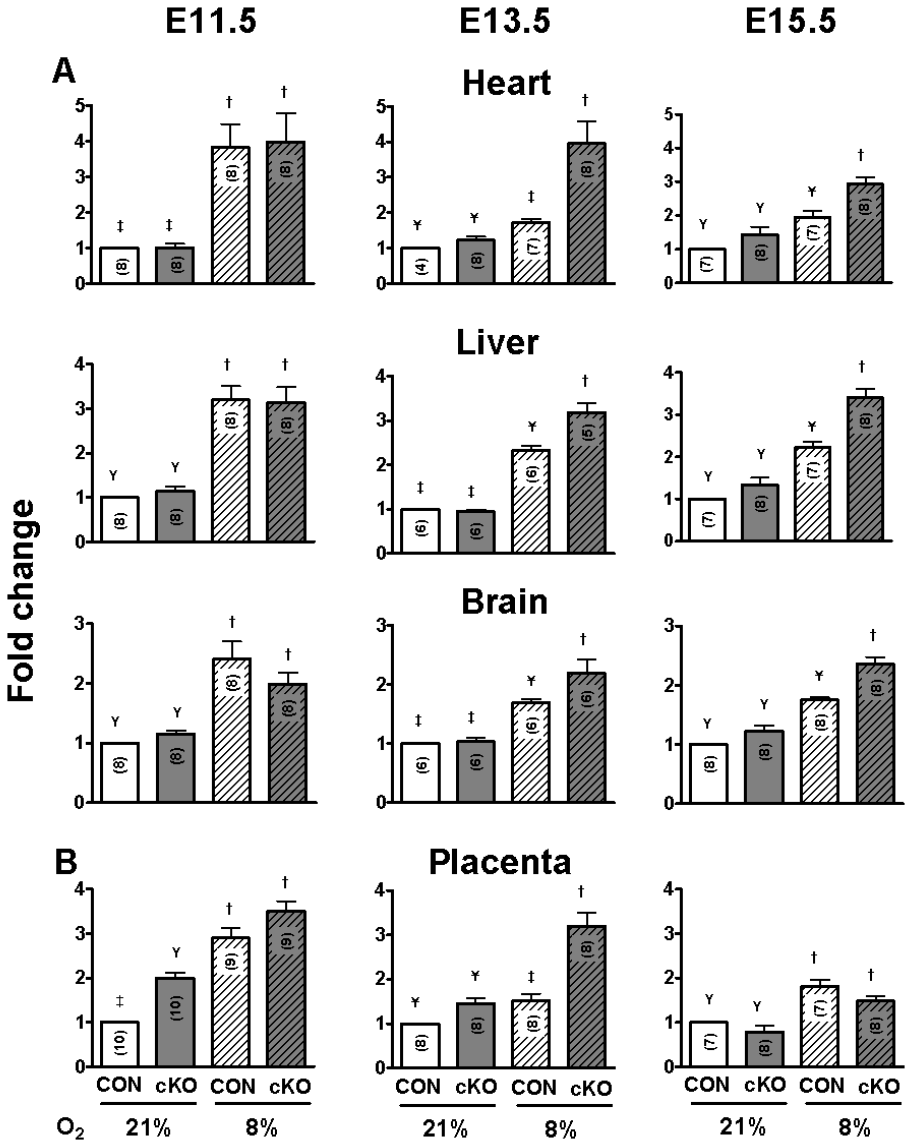
ODD-Luc mice underwent timed breedings and organs were harvested at the indicated days of gestation. Luciferase activity was measured in lysates from A) placenta B) heart C) liver and D) brain from E9.5- or 10.5 -17.5, at post-natal day 5 (PN5) and maturity (8 weeks). Pregnant ODD-Luc mice from the same stages were subjected to reduced concentrations of inspired oxygen (8% O_2 for 4 hours) and luciferase activity was measured. Luciferase activity was normalized to total protein and expressed as fmol Luciferase/mg protein. Data in dotted boxes in B, C, D are re-shown in insets with a compressed y-axis for clarity. The number of samples in each group is indicated within the bar graph (n). Student's *t*- test was used for comparison of hypoxia vs. room air samples. **P*<0.05; ***P*<0.01; ****P*<0.001. In the developmental series shown in A, B, C and D, basal luciferase activity (Room air) was analyzed by one-way ANOVA with Bonferroni correction for multiple comparisons; groups sharing the same symbol are not significantly different (*P*>0.05).

Figure 2. ODD-Luc activity is induced in E11.5 and E15.5 fetal tissues when dam's inspired O2 is reduced to 12%



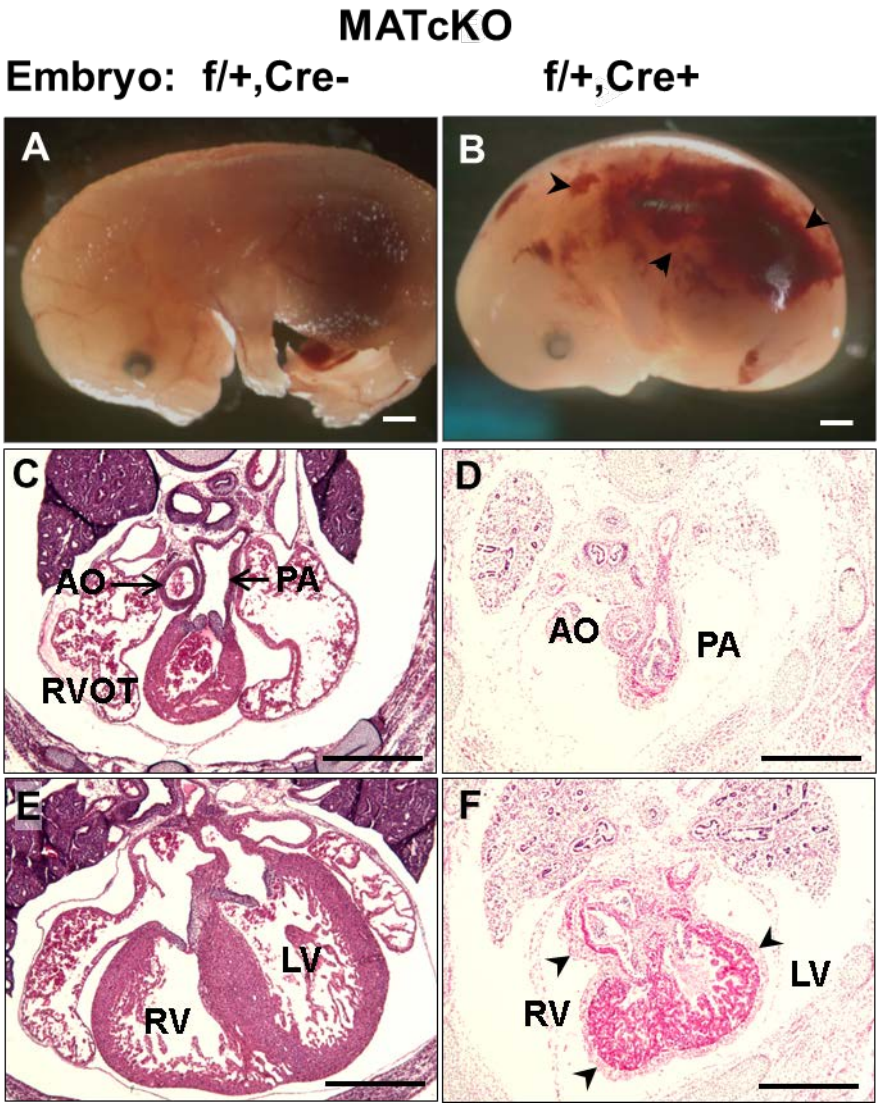
E11.5 and E15.5 timed pregnant females were exposed to 12% O2 for 8 hours. Gestational day matched control mice were exposed to room air (21% O2). Placental and fetal tissues were collected, luciferase activity was measured in lysates from heart, liver, brain and placenta. Luciferase activity was normalized to total protein and expressed as fold change compared to control. Values are mean ± SEM, n=8-11. Samples were analyzed by Student's t test. *P<0.05; **P<0.01; ***P<0.001.

Figure 3. Conditional knock-out of *Hif-1α* in maternal cells (MATcKO) reduces O_2 reserve at E13.5 and E15.5.



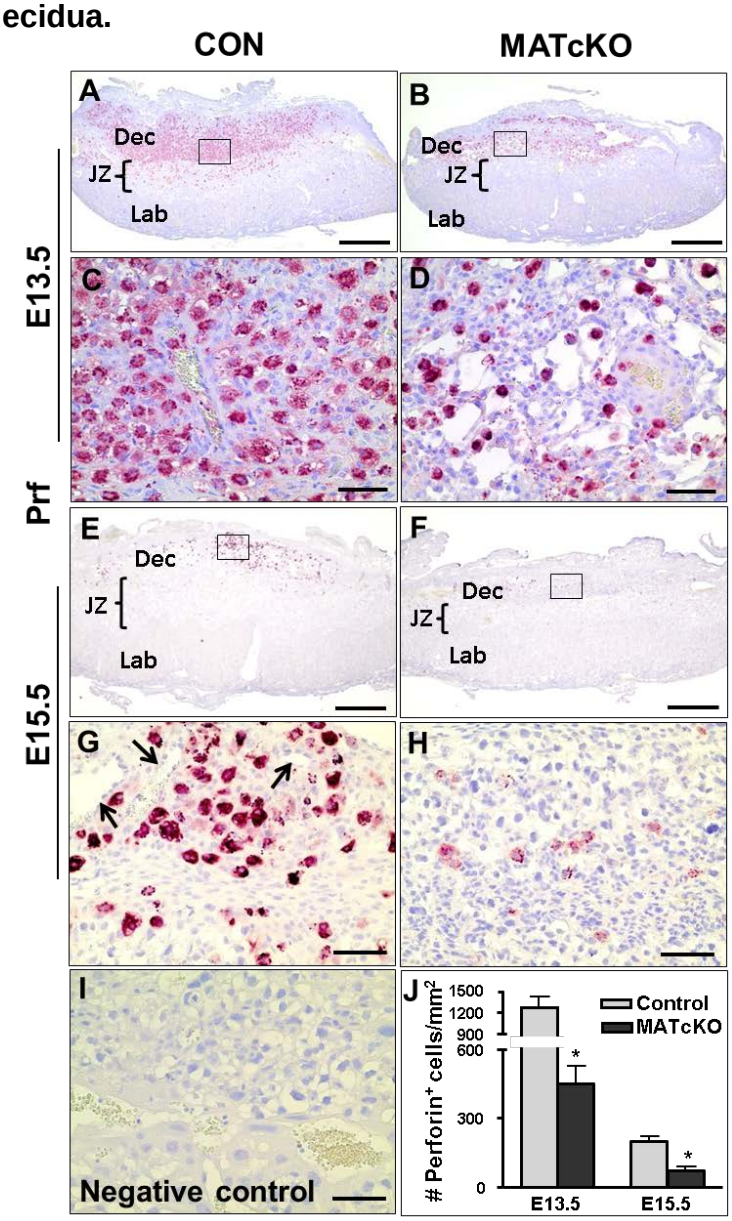
Hif-1α$^{f/f}$,*β-actinCre*$^{+}$ females were mated with ODD-Luc homozygous males and injected with Tamoxifen (3 mg/40 g bw) on E8.5 and 9.5. Control mice (*β-actinCre*$^{+}$) were mated with ODD-Luc males and treated with Tamoxifen in the same manner. One-half of the pregnant mice in each group were subjected to reduced concentrations of inspired O_2 (8% O_2 for 4 hours) at E11.5 or E13.5 or E15.5 and then euthanized. ODD-Luc activity per mg protein was measured in the placenta and fetal organs as described above. ODD-Luc activity in each organ at the gestational ages indicated was normalized to the value from the control mice under room air (21% O_2) and reported as fold-change. The number of samples in each group is indicated within the bar graph (n). Values in the different groups were compared by one-way ANOVA with Bonferroni correction for multiple comparisons; groups sharing the same symbol are not significantly different (P>0.05).

Figure 4. MATcKO *Hif-1α* results in fetal defects in *Hif-1α* heterozygous null embryos (*Hif-1α* $^{f/+}$,*Cre*$^{+}$).



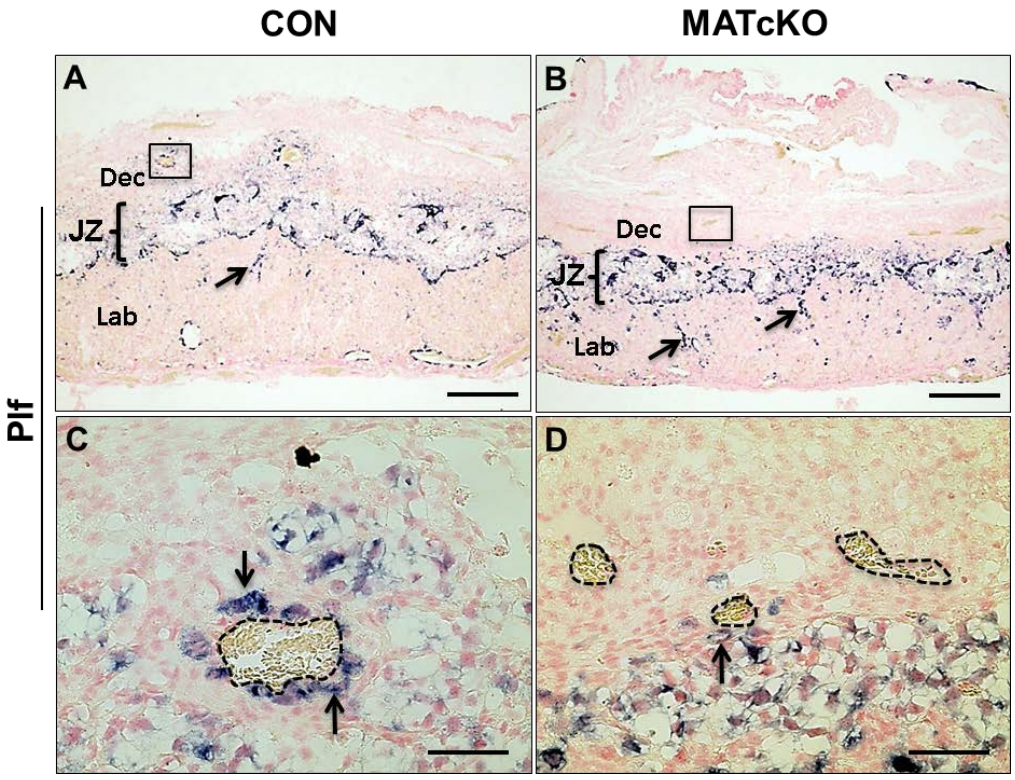
MATcKO *Hif-1α* was achieved as described in Fig. 1. Shown are embryos from a MATcKO dam that are effectively WT (*Hif-1α* $^{f/+}$,*Cre*$^{-}$) (A,C,E) versus *Hif-1α* heterozygous null (B,D,F). Embryos are shown in whole mount (A, B) and in H&E stained sections (C-F) from anterior to posterior with respect to the heart. The WT embryos have normal appearance in whole mount (A) and in sections (C, E). The *Hif-1α heterozygous null* embryos are smaller and edematous and can be seen to have hemorrhages (arrow heads in B). Embryos of this genotype were non-viable at E15.5. D, F) *Hif-1α* heterozygous null hearts were smaller, showed thickened epicardium (arrow head) and increased mesenchymal tissue in the septum. AO, Aorta; PA,

Figure 5. MATcKO *Hif-1α* causes reduction in uterine Natural Killer (uNK) cells in the placental decidua.



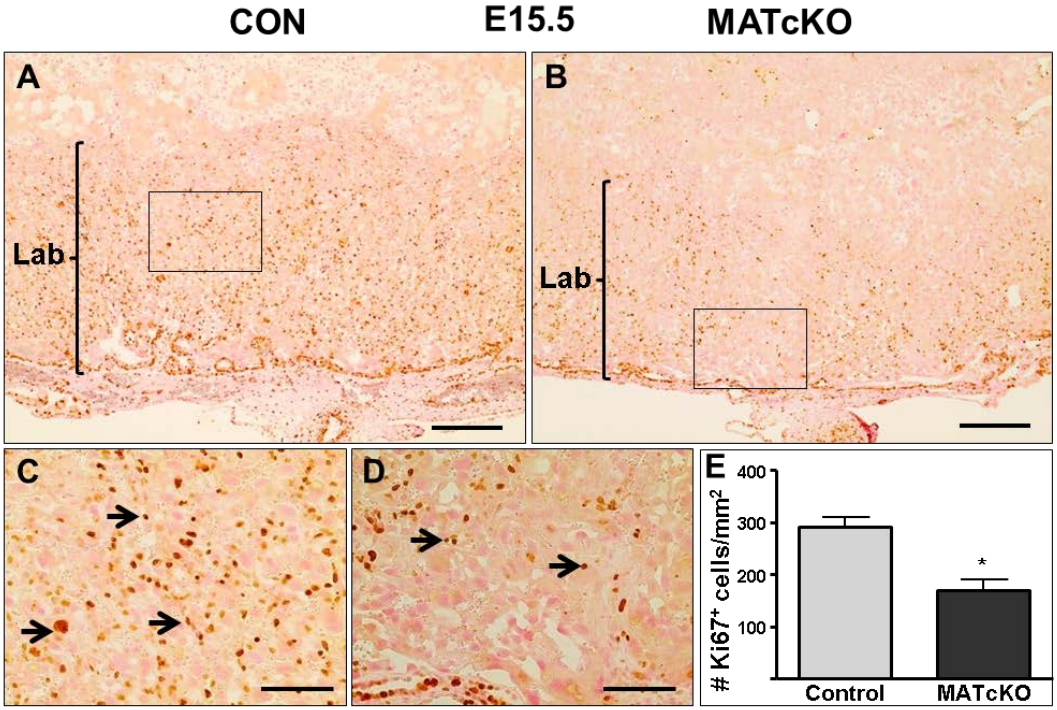
Maternally derived uNK cells were detected by immunostaining of E13.5 and E15.5 placentas using anti-Perforin antibody as described in Methods. The number of Perforin positive cells are significantly decreased at E13.5 and E15.5 in the decidua of MATcKO (B, F) as compared to CON (A, E). The boxed areas in A, B and E, F are shown at higher magnification in C, D and G, H respectively. Arrows in (G) indicate maternal decidual arterioles. J) The number of perforin positive cells were counted in 2-3 fields of view per section and presented as number (#) of perforin positive cells/mm^2. I) No primary antibody (negative control). Values are mean ± SEM *p<0.001, n=4-6. Scale bars (μm): A, B, E, F: 500; C, D, G - I: 50. Dec, decidua; Lab, labyrinth; JZ, junctional zone

Figure 6. MATcKO *Hif-1α* reduces trophoblast giant cell invasion of maternal decidua



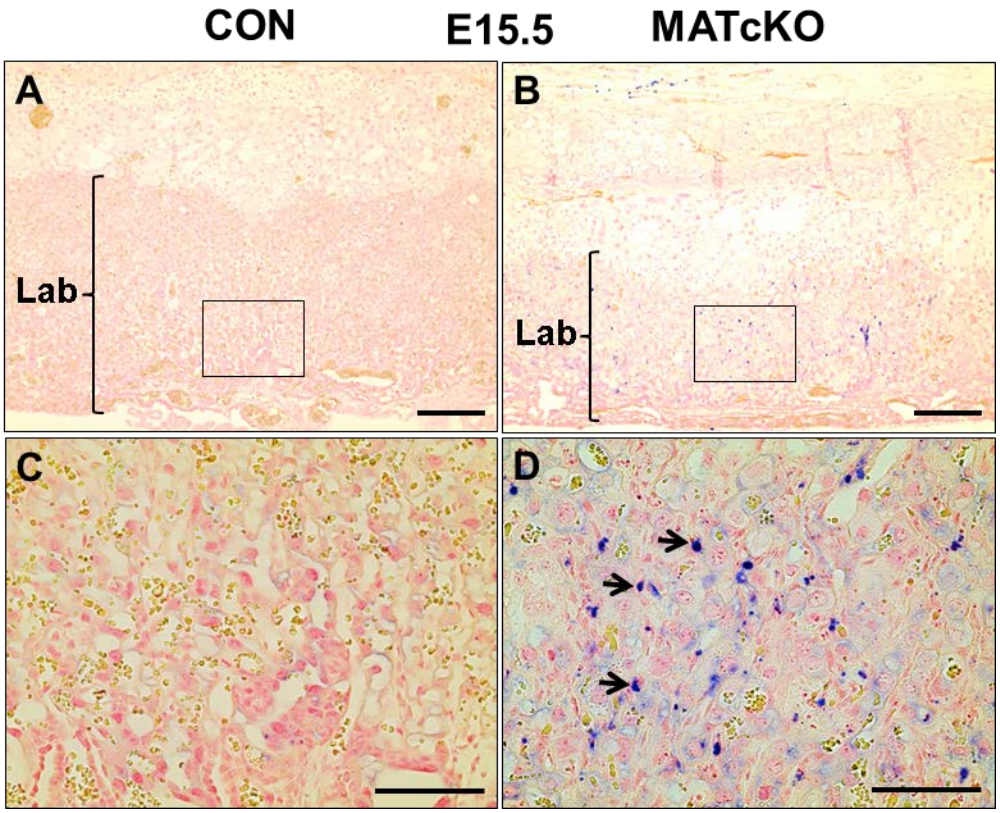
Sections from E15.5 CON and MATcKO placentas were examined by *in situ* hybridization for *Plf* (*Prl2c2*). *Plf* (*Prl2c2*)-expressing spiral artery-associated trophoblast giant cells (SpA-TGCs) are present in the junctional zone and decidua of CON placenta (A) and in the junctional zone of MATcKO, but are substantially reduced in the decidua of MATcKO (B). There are increased numbers of *Plf* (*Prl2c2*) positive cells in the labyrinth of MATcKO placenta (B, arrows). The boxed areas in A and B are shown at higher magnification in C and D where the Plf (*Prl2c2*) positive cells can be seen surrounding the spiral arterioles in the decidua of CON (C, arrows) but not in MATcKO (D). Dotted lines in C and D indicate maternal arterioles. Scale bars (μm): A, B: 500; C, D: 50. Dec, decidua; Lab, labyrinth; JZ, junctional zone

Figure 7. MATcKO *Hif-1α* reduced the number of Ki67 positive cells in E15.5 placental labyrinth.



Ki67 was detected by immuno-staining as described in Methods and used as an indicator of cell proliferation. The number of Ki67 positive cells are significantly decreased in the labyrinth of E15.5 MATcKO placentas (B) as compared to CON (A). The boxed areas in A, B are shown at higher magnification in C, D. Arrows in (C, D) indicate Ki67 positive cells. E) The number of Ki67 positive cells in the labyrinth were counted in one field of view per section and presented as number of Ki67 positive cells/mm^2. Values are mean ± SEM, *p<0.05, n=6 Scale bars (μm): A, B: 300; C, D: 100.

Figure 8. MATcKO *Hif-1α* induced TUNEL positive cells in E15.5 placental labyrinth.



Apoptosis was detected by the TUNEL assay which identified DNA fragmentation in E15.5 placentas. TUNEL positive cells are present in the labyrinth of MATcKO placentas (B) and not in CON (A). The boxed areas in A, B are shown at higher magnification in C, D. Arrows in (D) indicate TUNEL positive cells. Scale bars (μm): A, B: 300; C, D: 100.

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

  First, we demonstrate that the master regulator of transcriptional responses to hypoxia, HIF-1a, is required in the maternal cells for the normal development of the placenta. Our data supports a model in which hypoxia/Hif-1a in the maternal decidua is required for recruitment of uNK and TB cells into the decidua for the purpose of remodeling of the spiral arteries. When this fails, as in this model, the fetal heart and other tissues are rendered even more vulnerable to O2 deprivation. Second, we show gestational stage dependence of fetal vulnerability to O2 deprivation and suggest that this could lead to congenital heart defects, fetal failure-to-thrive and non-viability.

- **What was the impact on other disciplines?**

  'Nothing to report'

- **What was the impact on technology transfer?**

  'Nothing to report'

- **What was the impact on society beyond science and technology?**

  While we need to complete these studies, it suggests that even relatively modest acute increases in altitude, and concomitant reductions in inspired O2, could have adverse effects on the developing fetus and especially the fetal heart, particularly in pregnant women who are at risk due to placental abnormalities.

5. CHANGES/PROBLEMS

'Nothing to Report'

6. PRODUCTS

- **Publications, conference papers, and presentations**

Publications:

Kenchegowda D, Lemus M, Natale DR, Fisher SA, Conditional knockout of HIF-1a and ODDLuc hypoxia reporter to study O_2 in feto-placental development. **Developmental Biology** (Manuscript revised and resubmitted)

Conference papers and presentations:

Kenchegowda D, Lemus M, Natale B, Natale D, Fisher SA. Maternal conditional inactivation of HIF-1a causes placental insufficiency and compromises O2 delivery to the developing fetus under stress. Weinstein cardiovascular development conference, May 19th – May 21st 2016, Durham convention center, organized by Duke University of School of Medicine, Durham, NC USA.

Kenchegowda D, Fisher SA. cKO of HI-1a and ODDLuc hypoxia reporter to study feto-plaental development. Weinstein cardiovascular development conference, April 30th – May 2nd 2015, John B. Hynes Veterans Memorial convention center, organized by Boston Children's Hospita and Heart Center, Boston MA USA.

- **Website(s) or other Internet site(s)**

  'Nothing to Report'

- **Technologies or techniques**

  'Nothing to Report'

- **Inventions, patent applications, and/or licenses**

  'Nothing to Report'

- **Other Products:**

  'Nothing to Report'

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

➢ **What individuals have worked on the project?**

Name	Dr. Doreswamy Kenchegowda, PhD
Project Role	Postdoctoral Fellow
Researcher ID	F-2498-2012
Nearest person month worked	13
Contribution to project	Breeding, genotyping and maintenance of mouse lines, planning and conducting experiments, data analysis, writing manuscript, presentation of results in scientific meetings.
Funding support	DoD grant, award # W81XWH-15-1-0238

Name	Dr. David R. Natale, PhD
Project Role	Assistant Professor
Researcher ID	C-6810-2009
Nearest person month worked	3
Contribution to project	Placental immune-histochemical analysis and in situ hybridization assays, Data analysis, manuscript writing.
Funding support	Startup funds from Department of Reproductive Medicine, University of California, San Diego

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  'Nothing to Report'

- **What other organizations were involved as partners?**
  - **Organization Name:** Department of Reproductive Medicine, University of California, San Diego
  - **Location of Organization:** 2A03 Leichtag, Biomedical Research Building, 9500 Gilman Drive, MC 0674
  - **Partner's contribution to the project*:*** Laboratory facilities provided to Dr. David R. Natale
  - **Financial support:** Startup funds from Department of Reproductive Medicine, University of California, San Diego
  - **In-kind support*:*** none
  - **Facilities:** PCR machines, Microtome, Microscopes, reagents and kits required for in situ-hybridization and immunohistochemical analysis
  - **Collaboration:** Dr. David Natale, Dr. Bryony Natale and Ms. Maria Lemus
  - **Personnel exchanges:** none

8. SPECIAL REPORTING REQUIREMENTS

'Nothing to Report'

9. APPENDICES

'Nothing to Report'